Low-Molecular-Weight Solutes Released during Mild Acid Hydrolysis of the Lipopolysaccharide of *Pseudomonas aeruginosa*

IDENTIFICATION OF ETHANOLAMINE TRIPHOSPHATE

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A careful examination of the low-molecular-weight solutes released during mild acid hydrolysis of the lipopolysaccharide of *Pseudomonas aeruginosa* (N.C.T.C. 1999) revealed the presence of ethanolamine triphosphate. During storage, the compound decomposed to give ethanolamine pyrophosphate, identified in a previous study (Drewry *et al.*, 1971); PP_i may be a further decomposition product. Evidence for the attachment of ethanolamine triphosphate to a polysaccharide fraction was obtained, but the possibility that some was attached to the lipid A moiety was not excluded. Basic compounds released during the hydrolysis of lipopolysaccharide included amino acids, polyamines and oligopeptides.

Recently we reported the isolation of ethanolamine pyrophosphate (*O*-pyrophosphorylethanolamine; *O*-diphosphoethanolamine) from the lipopolysaccharide of *Pseudomonas aeruginosa* (Drewry *et al.*, 1971). This compound has also been obtained from the lipopolysaccharides of *Salmonella* (Osborn, 1969; Lüderitz, 1970; Mühlradt, 1971*a*), where it is linked to the 4-position of an aldoheptose residue (Lehmann *et al.*, 1971). In the lipopolysaccharide of *P. aeruginosa*, the linkage from ethanolamine pyrophosphate is apparently very acid-labile, as the compound is released during the mild acid hydrolysis used to separate the lipid A and polysaccharide moieties of the lipopolysaccharide. The possible existence in the lipopolysaccharide of higher condensed phosphates was suggested by the high release of P_i and the possible release of PP_i during the mild hydrolysis (Drewry *et al.*, 1971). To examine this possibility, hydrolysates have been prepared on a larger scale and additional precautions taken to preserve labile components during isolation procedures.

Materials and Methods

Growth of bacteria and preparation of cell walls

Cells of *P. aeruginosa* (N.C.T.C. 1999) were grown and cell walls prepared as described previously (Key *et al.*, 1970*a*; Drewry *et al.*, 1971).

Preparation, hydrolysis and fractionation of lipopolysaccharide

Lipopolysaccharide was extracted from cell walls by treatment with hot aqueous phenol and purified by high-speed centrifugation of the initial aqueous

extract (Key *et al.*, 1970*b*). Low-molecular-weight solutes released during mild hydrolysis (1% acetic acid at 100°C for 1 h) of the lipopolysaccharide were isolated by gel filtration followed by high-voltage electrophoresis (Drewry *et al.*, 1971). Each operation was carried out with minimum delay; components isolated were dissolved in deionized water and stored at –20°C.

Analytical methods

Total phosphorus was determined by the method of Bartlett (1959) and P_i by the method of Parvin & Smith (1969). 3-Deoxy-2-octulonic acid was determined by the method of Osborn (1963). Amino compounds were determined by using an automatic amino acid analyser (Technicon Instruments Co. Ltd., Basingstoke, Hants., U.K.).

Chromatographic and electrophoretic methods

Paper chromatography was carried out with Whatman papers (no. 1 for descending and no. 4 for ascending chromatography) and t.l.c. with layers of MN-300 cellulose (Macherey, Nagel and Co., Düren, Germany).

The following solvent systems were used: A, ethyl acetate–pyridine–water–acetic acid (5:5:3:1, by vol.); B, butan-1-ol–acetic acid–water (13:3:5, by vol.); C, propan-1-ol–aq. NH₃ (sp.gr. 0.88)–water (6:3:1, by vol.); D, butan-1-ol–pyridine–0.1 M-HCl (5:3:2, by vol.); E, dioxan–water–trichloroacetic acid–aq. NH₃ (sp.gr. 0.88) (65:27.5:5:0.25, v/v/w/v). High-voltage paper electrophoresis was carried out with Whatman papers (no. 1 for analytical and no. 3

for preparative work) and pyridine-acetic acid-water (5:2:43, by vol.; pH 5.3) as the buffer system.

Enzymic hydrolysis of phosphates

Solutions of phosphate esters in 0.05 M-(NH₄)₂CO₃ (pH 9.6) were covered by toluene and incubated with alkaline phosphatase (EC 3.1.3.1; type 1 from calf intestinal mucosa; Sigma Chemical Co., St. Louis, Mo., U.S.A.) for 16 h at 37°C. Samples of PP_i, dissolved in 0.05 M-tris-HCl buffer (pH 7.0) containing 1.3 mM-MgCl₂, were incubated with inorganic pyrophosphatase (EC 3.6.1.1; Boehringer und Soehne G.m.b.H., Mannheim, West Germany) for 16 h at 37°C.

Synthesis of ethanolamine triphosphate (*O*-triphosphoethanolamine)

The compound was prepared by the method described for ethanolamine pyrophosphate (Drewry *et al.*, 1971). A solution of Na₅P₃O₁₀ (197 mg) in water (18 ml) was adjusted to pH 7.5. Ethyleneimine (40 mg) was added and the mixture kept at 37°C for 40 h. After freeze-drying, the major ninhydrin-positive phosphorus-containing product (*m_p*, about 0.8) was isolated by preparative paper electrophoresis (pH 5.3). Analytical studies (described below) showed that this was the expected product, ethanolamine triphosphate.

Preparation of Dns derivatives

Peptides were treated with Dns chloride under the conditions described by Gray & Hartley (1963). After acid hydrolysis (6.1 M-HCl at 105°C for 4 h), Dns-labelled amino acids were separated by high-voltage electrophoresis at pH 5.3 and were detected by viewing the electrophoretograms under u.v. light and by spraying with ninhydrin (for basic derivatives).

Results and Discussion

As described previously (Drewry *et al.*, 1971), the low-molecular-weight solutes released by mild hydrolysis (1% acetic acid at 100°C for 1 h) of lipopolysaccharide could be fractionated by column chromatography on Sephadex G-10 and by high-voltage electrophoresis. When these operations were carried out with minimum delay, an additional ninhydrin-positive phosphorus-containing component was detected. On high-voltage electrophoretograms, this component had a mobility intermediate between that of P_i and that of ethanolamine pyrophosphate, and, during storage, the component gradually decomposed. This new component was also detected when the lipopolysaccharide was hydrolysed under slightly milder conditions (0.1 M-acetic acid at 100°C for 1 h).

The latter conditions have been used (Dröge *et al.*, 1970) to release a fragment containing 3-deoxy-2-D-manno-octulonic acid esterified with ethanolamine phosphate (*O*-phosphoethanolamine) in the 7-position, from the lipopolysaccharide (glycolipid) of a *Salmonella* Rd₂ mutant. Whereas this fragment had an electrophoretic mobility less than that of 3-deoxy-2-D-manno-octulonic acid, the present component from *P. aeruginosa* had a greater mobility and also failed to react with thiobarbituric acid reagent (Warren, 1960) or alkaline AgNO₃. To characterize this component, a large-scale hydrolysate was prepared from a fresh batch of lipopolysaccharide (1.26 g). Fraction D2 of the low-molecular-weight solutes (Drewry *et al.*, 1971) was isolated by chromatography on Sephadex G-10. The results of analysis of fraction D2 for amino compounds are given in Table 1; the values for amino acids are about three times those for the previous batch (Drewry *et al.*, 1971), but the molar ratios of the components for the two batches are very similar (Table 1). This suggests that the amino acids which are concentrated into fraction D2 do not arise through random contamination of the lipopolysaccharide. The presence of some galactosamine in fraction D2 shows that the chromatographic separation from polysaccharide was not quite complete.

Most of the individual components of fraction D2 were isolated by preparative high-voltage electrophoresis (Fig. 1), although certain of the fractions that were taken (5, 6, 7, 9) from this separation contained more than one component. The components of some fractions had been identified previously (Drewry *et al.*, 1971): fraction 5, free arginine, ornithine and lysine; fraction 8, ethanolamine phosphate; fraction 10, ethanolamine pyrophosphate. Of the additional components, fraction 11 was the new phosphorus-containing component, and fraction 12 was the component suspected previously to be PP_i (in the previous study, this component had been detected in fraction D3, which consisted mainly of free 3-deoxy-2-octulonic acid and P_i). The identification of fraction 12 as PP_i was confirmed by comparison with an authentic sample as follows: (a) high-voltage paper electrophoresis at pH 5.3; (b) t.l.c. on cellulose with solvent system E; (c) total hydrolysis to P_i by acid (1 M-HCl for 7 min at 100°C) or inorganic pyrophosphatase.

As noted above, fraction 11 decomposed during storage. Electrophoresis indicated that the initial decomposition products were P_i and ethanolamine pyrophosphate, whereas the products of vigorous acid hydrolysis (6.1 M-HCl at 105°C for 4 h) were ethanolamine phosphate, ethanolamine and P_i. As fraction 11 had an electrophoretic mobility greater than that of ethanolamine pyrophosphate, and in view of the occurrence of PP_i among the low-molecular-weight solutes, it seemed possible that

Table 1. *Amino compound composition of low-molecular-weight solutes in fraction D2 from the partly degraded polysaccharide*

Fraction D2 was obtained by consecutive chromatography on Sephadex G-75 and G-10 of partly degraded polysaccharide from the lipopolysaccharide of *P. aeruginosa* (Drewry *et al.*, 1971). Hydrolysis was carried out with 6.1 M-HCl at 105°C for 4 h. Results were obtained by autoanalysis and percentages are expressed as residues of amino compounds. Results in parentheses were obtained for fraction D2 from a previous batch of lipopolysaccharide (Drewry *et al.*, 1971).

	Content (% w/w)	Molar ratio	
Ethanolamine phosphate	9.38 (11.93)	—	—
Ethanolamine	1.70 (1.35)	—	—
Aspartic acid	3.16 (1.04)	0.37	(0.30)
Threonine	1.13 (0.31)	0.15	(0.10)
Serine	1.32 (0.45)	0.20	(0.17)
Glutamic acid	5.11 (1.61)	0.53	(0.42)
Glycine	0.96 (0.25)	0.22	(0.15)
Alanine	6.26 (1.90)	1.04*	(0.89)
Leucine	0.84 (0.29)	0.10	(0.09)
Tyrosine	1.19 (0.38)	0.09	(0.08)
Phenylalanine	0.19 (0)	0.02	(0)
Ornithine	1.56 (0.66)	0.18	(0.19)
Lysine	4.70 (1.42)	0.49	(0.37)
Histidine	0.46 (0.27)	0.04	(0.07)
Arginine	11.70 (4.69)	1.00	(1.00)
Galactosamine	1.21 (0)	—	—

* Corrected for contribution from contaminating polysaccharide.

Fig. 1. *Paper electrophoretogram of low-molecular-weight solutes in fraction D2 from the partly degraded polysaccharide*

The separation was carried out for 1 h at about 37 V/cm and pH 5.3. Spots were detected with either ninhydrin or the reagent of Hanes & Isherwood (1949) for phosphorus. Only fraction 12 did not react with ninhydrin; phosphorus was present in fractions 8–12 only.

with solvent systems A and B, and ascending chromatography with solvent system C; (d) hydrolysis (to ethanolamine and P_1) by alkaline phosphatase. In each case, fraction 11 behaved as ethanolamine triphosphate. To confirm the triphosphate residue, acid hydrolysis of the two phosphates was carried out under conditions commonly used to break pyrophosphate bonds, and the products were analysed. The results (Table 2) confirm the identification of fraction 11 as ethanolamine triphosphate. It should be noted that this compound was not separated from ethanolamine pyrophosphate by autoanalysis, so that the two compounds present in fraction D2 could not be determined separately by this means. However, it seems likely that much or all of the ethanolamine pyrophosphate was actually a decomposition product of the triphosphate.

Of the remaining components (Fig. 1), the highly mobile cationic fractions 1–3 (not detected in the previous study) were apparently di- or poly-amines. Such compounds occur commonly in bacteria (Cohen, 1971) and have been detected in lipopolysaccharide preparations (Lüderitz *et al.*, 1968). The electrophoretic mobilities of fractions 1, 2 and 3 corresponded to those of 1,3-diaminopropane, spermidine and cadaverine (and/or putrescine), respectively, but as only trace amounts of materials were available

fraction 11 was ethanolamine triphosphate or a higher homologue. Fraction 11 was compared with synthetic ethanolamine triphosphate as follows: (a) high-voltage paper electrophoresis at pH 5.3; (b) ion-exchange chromatography with the Technicon Auto-Analyser; (c) descending paper chromatography

Table 2. Acid hydrolysis of fraction 11 (Fig. 1) obtained from fraction D2 and synthetic ethanolamine triphosphate

Samples were hydrolysed with 1M-HCl at 100°C for 7 min. Percentage decomposition was determined by autoanalysis for amino compounds, and molar ratios for hydrolysis products were corrected for incomplete decomposition. Theoretical values for ethanolamine triphosphate are given in parentheses.

Sample	Decomposition (%)	Molar ratios of products	
		P_{total}/P_i	$P_i/\text{ethanolamine phosphate}$
Fraction 11	90	1.52	2.00
Synthetic product	88	1.40 (1.50)	2.18 (2.00)

Table 3. Properties of peptides present in fractions 6 and 7 (Fig. 1) obtained from fraction D2

Peptides were isolated from fractions 6 and 7 by preparative high-voltage paper electrophoresis for 2 h at 37 V/cm (pH 5.3)

Fraction	Electrophoretic mobility (m_{Arg})	Molar ratios							N-Terminal residue
		Ala	Arg	Asp	Glu	Gly	Lys	Ser	
6-1	0.86	1.16	—	—	—	1.00	1.87	—	Lys
6-2	0.78	1.18	1.11	—	—	1.00	1.00	0.89	Arg
6-3	0.75	1.12	1.00	—	—	—	—	0.73	Ala
6-4	0.72	2.22	1.00	—	—	—	—	—	Ala
6-5	0.67	1.93	1.00	—	0.78	—	—	—	Ala
7-1	0	1.00	—	0.78	1.03	—	—	—	Glu

Fig. 2. Release of low-molecular-weight solutes during mild acid hydrolysis of lipopolysaccharide

Lipopolysaccharide was hydrolysed with 1% acetic acid at 100°C. The hydrolysates were analysed for P_i (●), 3-deoxy-2-octulonic acid (○), ethanolamine phosphate (■), and ethanolamine pyrophosphate (□). Results for amino compounds are expressed in arbitrary units proportional to the peak areas obtained by autoanalysis of equal samples of hydrolysates. Results for P_i and 3-deoxy-2-octulonic acid are expressed as $\mu\text{g}/\text{mg}$ of lipopolysaccharide.

these tentative identifications could not be confirmed. Previous work (Chester *et al.*, 1972) on lipopolysaccharide from whole cells of *P. aeruginosa* had pointed to the presence of spermidine and putrescine; cadaverine (Stewart, 1970) and 2-hydroxyputrescine (Rosano & Hurwitz, 1969; Kullnig *et al.*, 1970) have been isolated from other strains of *P. aeruginosa*.

Fraction 4 was identified as ethanolamine by high-voltage paper electrophoresis, paper chromatography and autoanalysis. Fraction 6 contained a range of basic peptides with very similar electrophoretic mobilities: the peptides were separated by more prolonged electrophoresis (2 h at 37 V/cm). The compositions and N-terminal residues of the peptides are given in Table 3. The mobilities and amino acid compositions suggested that the peptides were small molecules (tri- to penta-peptides). Fraction 7 contained some free alanine (identified previously) and an almost neutral peptide (fraction 7-1 in Table 3). The near-neutrality of this peptide suggested that it contained residues of glutamine or asparagine, rather than the parent amino acids; the formation of a significant amount of ammonia during hydrolysis of the peptide supported this view.

The phosphorus-containing material (detected as

a streak after electrophoresis) in fraction 9 was apparently the residual galactosamine-containing polysaccharide noted earlier. Acidic peptides (giving discrete spots on either side of the phosphorus-containing streak) were also probably present in fraction 9, as it contained an enhanced amount of protein amino acids (mainly glutamic acid and aspartic acid). Whereas the presence of basic peptides, basic amino acids and polyamines in the lipo-

polysaccharide preparation may be explained in terms of counter-ions to the phosphate groups, the significance of the neutral and acidic peptides is not clear.

The extreme ease with which ethanolamine triphosphate was released by acid hydrolysis, and the instability of the compound, made it difficult to study the location or mode of attachment of the compound in the lipopolysaccharide. That the compound was covalently attached was indicated by the failure to

Fig. 3. Fractionation of partly degraded lipopolysaccharide on Sephadex G-75

Lipopolysaccharide (approx. 10mg) was hydrolysed with 1% acetic acid at 100°C for the stated periods (a, 10min; b, 35min; c, 1h). After treatment with chloroform, the aqueous hydrolysates were freeze-dried and solutions of the residues applied to a column (1cm×60cm) of Sephadex G-75. Elution was carried out with pyridine-acetic acid buffer (4ml of pyridine+10ml of acetic acid/l; pH 5.4) at a flow rate of 15ml/h. Fractions (2ml) were screened for total phosphorus by the method of Bartlett (1959).

Table 4. *Amino compound composition of fractions II and III (Fig. 3) from partly degraded polysaccharide*

Hydrolysis of samples was carried out with 6.1 M-HCl at 105°C for 4 h. Results were obtained by autoanalysis, and are expressed as $\mu\text{mol}/10\text{mg}$ of lipopolysaccharide (A, ethanolamine and ethanolamine phosphate; B, galactosamine) and as the molar ratio A:B.

Period of hydrolysis ...	Fraction ...	Analytical result					
		10min		35min		60min	
		II	III	II	III	II	III
A		0.040	0.045	0.113	0.077	0.129	0.094
B		0.170	0	0.544	0	0.740	0
A:B		0.24:1.00	—	0.21:1.00	—	0.17:1.00	—

detect it by high-voltage electrophoresis of unhydrolysed lipopolysaccharide. In an attempt to determine whether ethanolamine triphosphate was a true component of lipopolysaccharide or of a contaminating polyphosphate (Mühlrad, 1971a,b), and, if the former were the case, to determine whether it was bound to lipid or polysaccharide, mild hydrolyses were carried out for periods up to 70 min with 1% acetic acid at 100°C. The hydrolysates were analysed for P_i , 3-deoxy-2-octulonic acid and ethanolamine phosphates; the results are shown in Fig. 2. The significant values obtained at zero time (especially for 3-deoxy-2-octulonic acid and condensed phosphates) can probably be explained as a result of partial hydrolysis during dispersion of the lipopolysaccharide by sonication (45 min) in the 1% acetic acid. As shown in Fig. 2, ethanolamine phosphates were subsequently released more slowly than thiobarbituric acid-reactive 3-deoxy-2-octulonic acid, and the combined amount of ethanolamine pyrophosphate and ethanolamine triphosphate increased throughout the period studied. Thus, it seemed possible that these components might be detectable in lipid or polysaccharide fractions at intermediate hydrolysis times for the lipopolysaccharide. Samples of the aqueous phases from 10 min, 35 min and 1 h hydrolysates were fractionated by chromatography on Sephadex G-75; the results are shown in Fig. 3. As the period of hydrolysis increased, fraction I, which consisted mainly of relatively undegraded lipopolysaccharide (indicated by the presence of glucosamine, which occurs only in the lipid A moiety), was largely replaced by fractions II and III, corresponding to phosphorylated polysaccharide and low-molecular-weight solutes respectively (Drewry *et al.*, 1971). Fractions II and III were isolated and analysed for amino compounds (Table 4). As expected from Fig. 2, the amount of ethanolamine-containing compounds in fraction III increased as hydrolysis progressed. The same observation was made for fraction II, but the molar proportion of these compounds relative to galactosamine (a specific component of the polysaccharide)

decreased progressively. This result indicates that ethanolamine triphosphate is attached to the polysaccharide and is released at a rate lower than the rate of formation of polysaccharide fraction II. In apparent confirmation, no ethanolamine was detected in the chloroform-soluble lipid from any hydrolysate. However, as noted previously (Chester *et al.*, 1972), most or all of the thiobarbituric acid-reactive 3-deoxy-2-octulonic acid was released before any lipid A could be extracted into chloroform, and some glucosamine remained in the aqueous phase if hydrolysis was for less than about 30 min. One possible explanation for this result could be the presence in lipid A of hydrophilic residues (e.g. of ethanolamine triphosphate) that persist after hydrolysis of the ketosidic linkage from 3-deoxy-2-octulonic acid, which is expected to join the lipid and polysaccharide moieties. The occurrence of substantial amounts of phosphate residues in both lipid and polysaccharide could provide possible sites for the attachment of ethanolamine triphosphate residues to either moiety, by means of acid-labile pyrophosphate bonds. It should be noted, however, that the ester and pyrophosphate linkages of ethanolamine pyrophosphate attached to the 4-position of a heptose residue lacking free hydroxyl groups in the 3- and 5-positions are stable to hydrolysis by 0.1 M-acetic acid at 100°C for 1 h (Lehmann *et al.*, 1971). Attempts to demonstrate the release of ethanolamine triphosphate by amine-catalysed β -elimination, after periodate oxidation of the lipopolysaccharide (Osborn, 1969; Lehmann *et al.*, 1971), were inconclusive. Because of the small amount of the ethanolamine derivative and the presence of much carbohydrate and phosphorus in the reaction mixture, high-voltage electrophoretograms were unsatisfactory.

The biosynthesis of ethanolamine pyrophosphate residues in the lipopolysaccharide of *Salmonella minnesota* apparently involves initial transfer of the γ -phosphate residue from ATP to a heptose residue of the core polysaccharide (Mühlrad, 1971a). Subsequently an ethanolamine phosphate residue from

CDP-ethanolamine may be added to complete the biosynthesis (Lehmann *et al.*, 1971). Similar processes with intermediate steps leading to pyrophosphate (or higher condensed phosphate) residues can be envisaged for the biosynthesis of ethanolamine triphosphate residues in *P. aeruginosa*. It is, in fact, known that polyphosphate kinase occurs in the same fractions as enzymes involved in biosynthesis of lipopolysaccharide, and the formation *in vitro* of high-molecular-weight polyphosphates from ATP by such fractions has been demonstrated (Mühlradt, 1971*a,b*). However, in *S. minnesota*, such polyphosphates are not involved in the phosphorylation of heptose (Mühlradt, 1971*a*).

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